Studies on the Dermal Connective Tissue Barrier

BY

LENNART JUHLIN

UPPSALA 1956

ALMQVIST & WIKSELLS BOKTRYCKERI AB

FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF UPPSALA, SWEDEN

Studies on the Dermal Connective Tissue Barrier

AKADEMISK AVHANDLING

som med tillstånd av Medicinska Fakulteten vid
Uppsala Universitet för ernående av medicine doktorsgrad
offentligen försvaras å Farmakologiska Institutionen
fredagen den 2 november 1956 kl. 10 f.m.

av

LENNART JUHLIN
Med. lic., Vg.

UPPSALA 1956
ALMQVIST & WIKSELLS BOKTRYCKERI AB

This paper is a summary of results published in the following articles.

I. Edlund, T. & L. Juhlin: Studies on the permeability of connective tissue. I. The effect of dibenamine on the decreased dermal spread caused by intra-articular burns, corticotropine, posterior pituitary extract, adrenaline, and noradrenaline. *Acta pharmacol. et toxicol.* 1954, **10**, 390.

II. Edlund, T. & L. Juhlin: Studies on the permeability of connective tissue. II. The effect of antihistamines, antiadrenergic agents, hexamethonium, and some theophylline derivatives on dermal spread. *Ibid.* 1955, **11**, 37.

III. Juhlin, L.: The role of oedema in the clearance of Evans blue and haemoglobin from inflammatory foci. *Acta path. microbiol. Scand.* 1956, **38**, 385.

IV. Juhlin, L.: Reconstitution of dermal connective tissue barrier after testicular or bacterial hyaluronidase. *Acta pharmacol. et toxicol.* 1956, **12**, 96.

V. Juhlin, L.: Studies on the dermal connective tissue barrier and its reconstitution after administration of testicular and bacterial hyaluronidase. The effect of cortisone, protamine, heparin, promethazine, and compound 48/80. *Ibid.* 1956, **12**, 321.

VI. Juhlin, L.: Studies on the dermal connective tissue barrier of alloxan-diabetic rabbits and its reconstitution after adminstration of testicular and bacterial hyaluronidase. *Acta Derm.-Venereolog.* 1956, **36**, 85.

VII. Juhlin, L.: The spreading of spherical particles in dermal connective tissue. *Ibid.* 1956, **36**, 131.

References to these papers will be made by using the Roman numerals, as above.

Introduction

Working indepdendently McClean (1930) and Hoffman and Duran-Reynals (1930) found that the area of spread of Indian ink injected intradermally was greatly increased by the addition of testicular extract. It is now known that the barrier overcome by the extract consists of a protein–mucopolysaccharide complex; and that the testicular extract contains the enzyme hyaluronidase which degrades and liquefies the mucopolysaccharide part, chiefly hyaluronic acid. Hyaluronidase is usually prepared from testicular tissue or bacteria, and is also found in leeches and in snake and insect venoms. The effect of the enzyme on

the spreading of bacteria, virus, and cancer was reviewed by Duran-Reynals (1942). During recent years the study of connective tissue has come more and more to the fore. The technique of injecting a coloured indicator into the skin and measuring its area of spread has been used by a number of investigators as a measure of the permeability of connective tissue. The effect of hormones and drugs has been examined in this way. In assessing the findings of such investigations it must be borne in mind that the area of spread is determined by several factors.

In experiments on living animals the extent of spreading is determined by the state of the connective tissue proper during the first few minutes only. Such factors as vascular pulsations, movement of oedema fluid, and absorption from the site of injection rapidly become of greater significance. The effect of these secondary factors on intradermal spreading is examined in papers I–III.

In order to study the physiology and pharmacology of the connective tissue proper by spreading-experiments these secondary factors must be eliminated: such experiments are reported in papers IV–VII.

Personal Investigations

Paper I

The aim of this investigation was to study in living rabbits the effect on dermal spread of remote injury and hormones claimed to be released during the earlier phases of Selye's general adaptation syndrome (GAS). As indicator a solution of human haemoglobin was injected into the skin of the back of depilated living rabbits, and its spreading area measured 15, 30, 45, and 60 minutes later. The use of Indian ink as indicator was shown to produce inflammation, which could constitute a source of error. Thus the decreased spread after administration of adrenal cortical hormones reported by Opsahl (1949) might be explained by their anti-inflammatory action. When haemoglobin was used as indicator, however, desoxycorticosterone acetate, cortisone acetate, and corticosterone had no influence on dermal spread when injected shortly before the test (Edlund and Juhlin, unpublished).

Animals exposed to stress by injecting 70° Ringer solution intra-articularly showed diminished spreading of haemoglobin injected intradermally 30 minutes later. The effect was probably not due to the cortical hormones, since desoxycorticosterone acetate, cortisone acetate, and corticosterone were without effect, as stated above. ACTH reduced the spreading, but only in proportion to its content of vasopressin.

When spreading is measured in living animals 15–60 minutes after injection of indicator one would expect it to be markedly influenced by the state of the circulation in the small blood vessels, and particularly by their pulsations (McMaster and Parsons, 1938). Our working hypothesis was then that an alarm reaction induced by injury leads to vasoconstriction in the skin through an adrenergic

mediator. Evidence for this was obtained in experiments in which we showed that both adrenaline and noradrenaline decreased spread to the same degree as intra-articular burns, and that the decreased spread could be restored to normal by an adrenergic blocking agent, dibenamine®, in 'burned' animals and animals treated with adrenaline and noradrenaline. The decreased spread found after administration of ACTH or vasopressin could not be brought to normal by dibenamine, which makes it less probable that the decreased spread found after injury was mediated by vasopressin. Since, however, the specificity of dibenamine as an adrenergic blocking agent in spreading-experiments may be doubted (II), it cannot be considered as definitely proved that the decreased spread of 'burned' animals is due to an adrenergic mediator. In any case the decreased spread found in the experiment seemed to be due to vasoconstriction of the vessels of the skin.

Paper II

In paper I it was found that the spreading of haemoglobin could be decreased by drugs causing vasoconstriction. In paper II the effect of vasoactive drugs on dermal spread is further examined. Mayer and Kull (1947) found that antihistamines (antazoline and tripelennamine) decreased dermal spread, and concluded that their therapeutic effects in some allergic states might partly be due to inhibition of hyaluronidase. It was thought worth while to investigate whether or not antihistamines could influence spreading by affecting the vessels.

Our indicator solutions contained no measurable amounts of histamine, and we found that antazoline, 3015 RP, and phenindamine decreased dermal spread, whereas two other antihistamines, both more potent than antazoline, increased spread (Sc 1742) or did not influence it (Sy 14). All the effects were abolished by dibenamine. From these findings it was concluded that the antihistamines influenced dermal spread by mechanisms other than their antihistaminic action. No evidence was found that hyaluronidase was involved in the altered spreading due to antihistamines.

Yet another antihistamine, diphenhydramine-8-chlorotheophylline (Dramamine®), increased spread, an effect which was found to be due to the 8-chlorotheophylline molecule. Theophylline–isopropanolamine too increased the spreading of haemoglobin, and the effects could be abolished by dibenamine. The effects were thought to be due to peripheral vasodilatation of the blood vessels. The same was true of the increased spread caused by hexamethonium.

Haley and Harris (1949) studied the circulatory effect of certain drugs on the vessels of the rat mesoappendix. Some of the drugs studied by these authors were the same as those used here. It was found that those designated by Haley and Harris as vasodilators increased spreading, and that the vasoconstrictors diminished spreading. Some of the antihistamines and theophylline derivatives were also tested on recently-killed animals, and did not influence spread. It there-

fore seems probable that the effect of the compounds tested is due to vasoconstriction or vasodilatation in the rabbit's skin.

In experiments where antazoline, 3015 RP, and its methachloride (Sc 1742) were injected dissolved in the haemoglobin solution they increased and decreased spread just as when injected intravenously. This indicates that they must act topically. The fact that the effects of 3015 RP injected intravenously could not be prevented by intravenous injection of hexamethonium, which itself increased spread, is also in favour of a local action. That dibenamine brought back to normal both the increased and decreased spread indicated that its effect was due to actions other than its adrenolytic and sympathicolytic properties.

Paper III

In the literature there are contradictory results regarding spreading and absorption from inflammatory foci. The role of inflammatory oedema in the spreading (local invasiveness) and the entry of agents from a localized lesion into the systemic circulation (general invasiveness) was therefore studied in the skin of the pinna of unanaesthetized rabbits. Different degrees of inflammation and oedema were produced in the pinna by burning or injection of *Staphylococcus aureus*. The spreading-indicators, haemoglobin or Evans blue, were then injected into the inflamed area at the same time or after 2 or 24 hours. The degree of oedema was estimated simultaneously with the extent of the spreading by measuring the thickness of the ear by the absorption of β-rays from P^{32}.

Spreading from the inflammatory focus was greatly increased in fresh and 2-hour lesions, and in some 24-hour lesions. When spreading took place during the development of oedema the growth of the stained patch kept pace with that of the oedematous area. When dye was injected into an already existing inflammatory focus it spread rapidly, but seldom reached the outer limit of the oedema. A strongly positive correlation was found between the area of spread and the degree of oedema measured at the same time. In most of the inflammatory foci a central decolorization developed. The dyed patch became ring-shaped and, in some cases, later crescent-shaped.

A factor which may have contributed to the increased spreading from an inflammatory focus is an increase in vascular pulsation. Marked vasodilatation without inflammation, as caused by warming of the pinna, resulted in only a small increase in spread, however, and the phenomenon of central decolorization could not be explained by an increase in vascular pulsation.

The rate of removal of dye from the inflamed region via the lymph vessels was raised during the first hours of inflammation, and sometimes even in 24-hour lesions. Menkin's theory (1956) of walling off with diminished absorption owing to formation of thrombi received no support.

All findings are in keeping with the concept that within an area of inflammation

a steady state exists between the focal production of oedema fluid and its absorption perifocally. The centrifugal flow of fluid within such an area is responsible for the increased spread.

*

In the experiments reported in the following papers some aspects of the physiology and pharmacology of the dermal connective dissue proper were studied in newly-killed animals.

Paper IV

This investigation was made to establish how rapidly the tissue barrier is reconstituted in vivo after breakdown with hyaluronidase, and whether or not the barrier built up again after the process of breakdown has the same properties as has intact tissue. In all previous investigations into the connective tissue barrier the testicular enzyme has been employed. Since, however, the bacterial enzyme occurs commonly in the course of natural pathological processes and has different properties, a systematic comparison was made between the enzymes.

The spreading-area of haemoglobin with or without supramaximum doses of hyaluronidase injected immediately after death was examined in control skins and skins pretreated in vivo with hyaluronidase.

Six hours after breakdown by bacterial hyaluronidase the residual effect had fallen to rather less than half of the initial effect, but the pretreated area was totally unresponsive to both bacterial and testicular enzyme. A tissue barrier completely resistant to hyaluronidase had thus been formed against spreading. This barrier disappeared within 24 hours, when spreading in the pretreated area was practically restored to normal, and the tissue had regained full sensitivity to hyaluronidase.

The residual effect remaining 6 hours after similar treatment with testicular hyaluronidase was less than that seen after bacterial enzyme, and subsequent injection of hyaluronidase into the pretreated area increased spreading to some extent. A hyaluronidase-insensitive barrier was also present, however. After 24 hours the situation was the same as with bacterial enzyme.

Paper V

Factors influencing the connective tissue barrier and its reconstitution after supramaximum doses of bacterial and testicular hyaluronidase were further studied. Again all measurements of spreading were made on newly-killed animals.

The loose connective tissue is regarded as a collagen network filled with ground-substance. A change in the degree of spreading of haemoglobin alone might be due to a change in either or both of these components. If haemoglobin is injected with surpramaximum doses of hyaluronidase the extent of spreading will no longer

be limited by the hyaluronic acid in the ground-substance, since this component is completely broken down. Under these conditions the remaining hyaluronidase-insensitive material and the collagen network will be the factors limiting spreading.

The spread of haemoglobin alone was decreased by cortisone treatment over 3 days, cortisone + protamine, and promethazine. The spread of haemoglobin + enzyme was decreased only by promethazine, and was increased by heparin and by a single injection of cortisone. These two latter treatments had no effect on the spread of haemoblogin alone. From this it is clear that there existed a lack of parallelism between the effects of various treatments in vivo on post-mortem spreading of haemoglobin with or without hyaluronidase.

The maximum spreading effect of testicular hyaluronidase was increased by cortisone in 'acute' experiments, and by heparin. This could be explained if these drugs act by preventing a swelling of the fibril bundles of the collagen network, or by diminishing the rate with which this swelling takes place after breakdown of a presumed mucopolysaccharide cement holding the bundle together.

The hyaluronidase-insensitive barrier formed after breakdown of the mucopolysaccharides in the skin by bacterial hyaluronidase was found to be more marked if the animals were treated with heparin or cortisone on the same day. As a possible explanation of the formation of the hyaluronidase-insensitive barrier and its increase with heparin it is proposed that heparin forms a mucopolysaccharide–protein complex, and that this hyaluronidase-insensitive complex replaces the normal complex containing hyaluronic acid. The effect of cortisone may be due to the liberation of heparin, since there is evidence in the literature that cortisone degranulates the mast cells (Wegelius and Asboe-Hansen, 1956), and that the mast cells yield heparin and other substances on degranulation (Riley, 1955).

When the animals were given cortisone 48 and 24 hours previously, in addition to the dose on the day of the experiment, no increase in the newly-formed hyaluronidase-insensitive barrier was seen. The reason for this may be that the heparin stores in the mast cells had fallen by the day of the experiment, so that at the time of the final injection of cortisone less heparin was available to be liberated into the skin. That a single dose of cortisone acts by liberating heparin is further supported by the fact that the increase in the enzyme-insensitive barrier was prevented if protamine was exhibited at the same time as cortisone.

Experiments were also performed on skin in which an attempt had been made to deplete the mast cells by pretreatment with compound 48/80. The residual effect after hyaluronidase pretreatment was in this way strongly decreased. No explanation is offered for this.

Throughout the experiments reported in papers IV and V characteristic differences were noted between the effects of bacterial and testicular hyaluronidase. These may have been due to the fact that the two enzymes break down hyaluronic acid by different actions. If the less depolymerized and changed breakdown

products resulting from pretreatment with the testicular enzyme have a greater affinity for the protein-scaffolding left after breakdown of the ground-substance than those resulting from the action of bacterial enzyme, most of the differences observed can be explained.

Paper VI

The high incidence of localized necrotic and suppurative processes in diabetic skin has been thought to be due to an increased content of glucose in the skin, providing a better substrate for bacterial growth (Urbach, 1946). The connective tissue barrier is also of importance in the spreading of infections (Duran-Reynals, 1942). It was therefore investigated whether the connective tissue barrier or its reconstitution after breakdown by hyaluronidase are different in the skin of alloxan-diabetic animals.

The spread of haemoglobin alone in intact skin was the same in diabetic and control animals. The spreading of haemoglobin + hyaluronidase was not significantly different, though it was slightly greater in the diabetic animals. In areas pretreated with bacterial hyaluronidase a hyaluronidase-sensitive barrier had formed after 6 hours in diabetic animals, whereas in the controls no enzyme-sensitive barrier was formed. The hyaluronidase-sensitive barrier normally formed after injection of testicular enzyme was greater in diabetic animals, but the enzyme-insensitive barrier was the same in diabetic and control animals.

Schiller and Dorfman (1955) were able to isolate less hyaluronic acid and chondroitinsulphuric acid from the skin of alloxan-diabetic rats than from controls. Their method was not suitable for quantitative determinations of the total amounts present, however. The amount of hexosamine was therefore estimated in dried skin. No difference was found between diabetic and control rabbits. From experiments in which the hyaluronic acid and chondroitinsulphate A in the skin had been reduced by pretreatment with hyaluronidase it was concluded that at least about 25 % of the hexosamine was derived from these mucopolysaccharides. If such a marked diminution of the amount of hyaluronic acid as noted in diabetic rats by Schiller and Dorfman (1955) had occurred it would have been revealed by the hexosamine determinations.

No correlation could be found between nitrogen content per sq. cm skin and spread of haemoglobin with or without hyaluronidase.

Paper VII

In the previous papers of this series spreading-indicators of molecular size were used. In paper VII the spreading of larger particles was studied.

Spherical particles marked with a fluorescent dye were prepared by emulsion-polymerization of methyl methacrylate. The influence of size and wettability of the particles on their spreading in connective tissue was studied in normal skin

and skin pretreated with bacterial hyaluronidase. The mean diameters of the particles used were 0.1 μ and 0.75 μ. Both hydrophilic and lipophilic particles were prepared by using different surface-active agents as emulgators.

The spreading-area of 0.1-μ particles was greatly increased by hyaluronidase, and no difference was noted between hydrophilic and lipophilic particles. A hyaluronidase-insensitive barrier developed against the spread of 0.1-μ particles in areas pretreated with the enzyme 6 hours earlier. During spreading the particles were filtered off from the suspending glucose solution, and they migrated independently of it.

Injection into intact tissue of particles with a mean diameter of 0.75 μ was followed immediately by filtering-off of the particles and continued spreading of the glucose solution only. The spreading-area of the particles was less if the injection was performed more slowly than usual. When the 0.75-μ particles were injected into enzyme-treated skin an increase in the area of spreading was noted if the particles were hydrophilic, but a decrease if they were lipophilic. The lipophilic particles, however, had a tendency to aggregate, making it uncertain whether the difference in spreading was due to a difference in their wetting properties as such. No enzyme-insensitive barrier could be demonstrated with either hydrophilic or lipophilic 0.75-μ particles.

The spreading-area of the 0.75-μ particles probably depends chiefly on the extent of a central cavity formed by disruption during the injection. The decreased spreading of particles on slow injection can be explained by outward streaming of fluid into the peripheral tissue, resulting in a lesser disruption. Similarly, if the tissue resistance to streaming fluid was impaired by treatment with hyaluronidase, reduction in the size of the central cavity might follow. Thus it might be explained why a smaller spreading-area was obtained with readily-aggregating lipophilic particles measuring 0.75 μ in enzyme-treated tissue than in intact tissue. That the spreading-area of hydrophilic 0.75-μ particles increased in enzyme-treated tissue indicated that these were able to enter a zone surrounding the central cavity.

The functional size of the pores limiting lateral spread through hyaluronidase-treated skin is estimated to lie between 0.1 and 0.6 μ.

ACKNOWLEDGEMENTS

I am indebted to Torbjörn Edlund, M.D., who introduced me into this field of research.

I wish to thank Professor Ernst Bárány, M.D., for all his advice and stimulating interest in my work.

Thanks are also due to Mrs. Asta Palis for valuable technical assistance and to Marcia Skogh, M.B., Ch.B., for translating the Swedish text.

REFERENCES

Duran-Reynals, F.: *Bact. Rev.* 1942, **6**, 197.

Haley, T. J. and D. H. Harris: *J. Pharmacol.* 1949, **95**, 293.

Hoffman, D. C. and F. Duran-Reynals: *Science* 1930, **72**, 508.

Mayer, R. L. and F. C. Kull: *Proc. Soc. Exp. Biol. & Med.* 1948, **67**, 529.

McClean, D.: *J. Path. Bact.* 1930, **33**, 1045.

McMaster, P. D. J. and R.J. Parsons: *J. Exp. Med.* 1938, **68**, 337.

Menkin, V.: Biochemical Mechanisms in Inflammation. C. C. Thomas Publ. Springfield, Ill. 1956.

Opsahl, J. C.: *Yale J. Biol. & Med.* 1949, **21**, 487.

Riley, J. F.: *Pharmacol. Rev.* 1955, **7**, 267.

Schiller, S. and A. Dorfman: *Biochem. Biophys. Acta*, 1955, **15**, 304.

Urbach, E.: Skin Diseases Nutrition & Metabolism. Grune & Stratton, N.Y. 1946.

Wegelius, O. and G. Asboe-Hansen: *Acta Endocrinolog.* 1956, **22**, 157.